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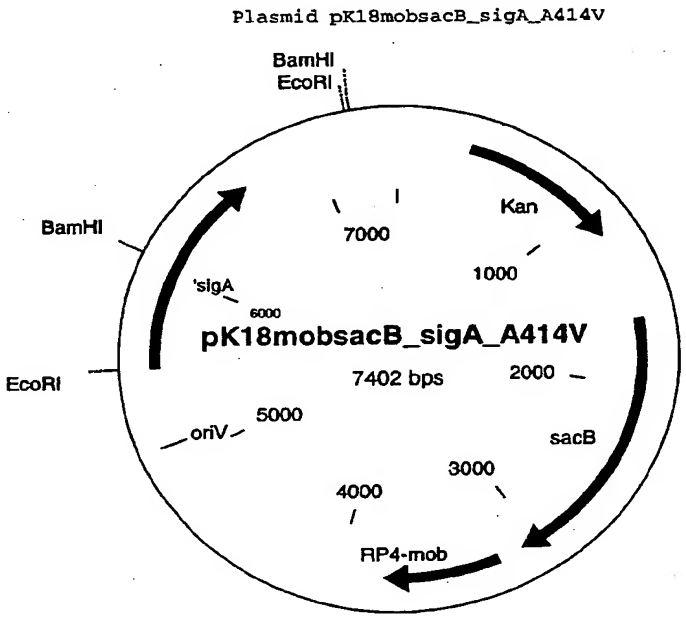
(54) Title: ALLELES OF THE SIGA GENE FROM CORYNEFORM BACTERIA



(57) Abstract: The invention relates to alleles of the sigA gene from coryneform bacteria which code for sigma factors A and a process for the fermentative preparation of L-lysine using bacteria which contain these alleles.

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Alleles of the sigA gene from coryneform bacteria

Field of the Invention

The invention provides alleles of the sigA gene from coryneform bacteria which code for variants of sigma factor A and a process for the fermentative preparation of L-lysine using bacteria which contain these alleles.

Prior art

The amino acid L-lysine is used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner. A known antimetabolite is the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC).

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by

amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

The nucleotide sequence of the gene which codes for sigma factor A from Corynebacterium glutamicum can be found in the patent application EP-A-1108790 as sequence no. 2100 and as sequence no. 7065.

The nucleotide sequence is also deposited in the databank of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA) under Accession Number AX122184 and under Accession Number AX127149.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of L-lysine.

Summary of the invention

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

The invention provides replicatable nucleotide sequences (DNA) which originate from coryneform bacteria, in particular Corynebacterium glutamicum, and code for sigma factor A, wherein the associated amino acid sequences in SEQ ID No. 2 contains any proteinogenic amino acid excluding L-alanine at position 414.

The invention furthermore provides a replicatable nucleotide sequence (DNA) which originates from coryneform bacteria, in particular Corynebacterium glutamicum, and codes for sigma factor A, wherein the associated amino acid sequence contains L-valine at position 414, shown in SEQ ID No. 4.

The invention furthermore provides a replicatable nucleotide sequence (DNA) which originates from coryneform bacteria, in particular Corynebacterium glutamicum, and codes for sigma factor A, the base sequence of which contains thymine at position 1241, shown in SEQ ID No. 3.

The invention furthermore provides plasmids (vectors) which contain the nucleotide sequences according to the invention and optionally replicate in coryneform bacteria.

The invention furthermore provides coryneform bacteria which contain the nucleotide sequences according to the invention and in which the nucleotide sequences which code for sigma factor A are optionally present in over-expressed form, wherein the associated amino acid sequences contain another proteinogenic amino acid at position 414 of SEQ ID No. 2.

Over-expression is understood as meaning an increase in the intracellular concentration or activity of the sigma factors A according to the invention.

By over-expression measures, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the activity or concentration of the protein in the starting microorganism.

Sigma factor A is a transcription factor which mediates the binding of RNA polymerase to specific sites (initiation sites) of the DNA and initiates the start (initiation) of transcription. It participates in the initiation of transcription a large number of genes, for example the genes hom, which codes for homoserine dehydrogenase, gap, which codes for glyceraldehyde 3-phosphate dehydrogenase, fda, which codes for fructose bisphosphate aldolase, and

pgk, which codes for phosphoglycerate kinase (Pátek et al., Microbiology 143: 1297-1309 (1996).

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-lysine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Plasmids which are replicated in coryneform bacteria are suitable for increasing the number of copies of the sigA alleles according to the invention. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891) can be used in the same manner.

The method of chromosomal gene amplification, such as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon, can

furthermore be used to increase the number of copies. In this method, the complete gene or allele is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman, Journal of Biological Chemistry 269:32678-84 (1994); US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al., Journal of Bacteriology 173:4510-4516 (1991)) or pBGS8 (Spratt et al., Gene 41: 337-342 (1986)). The plasmid vector which contains the gene or allele to be amplified is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene or allele in question.

The increase in protein concentration is detectable via 1- and 2-dimensional protein gel separation and subsequent optical identification of the protein concentration in the gel with appropriate evaluation software. A common method for preparation of the protein gels in the case of coryneform bacteria and for identification of the proteins is the procedure described by Hermann et al. (Electrophoresis, 22:1712-23 (2001)). The protein concentration can also be analysed by western blot hybridization with an antibody specific for the protein to be detected (Sambrook et al., Molecular cloning: a

laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) and subsequent optical evaluation with appropriate software for determination of the concentration (Lohaus and Meyer (1998) Biospektrum 5:32-39; Lottspeich (1999) Angewandte Chemie 111:2630-2647). The activity of DNA-binding proteins can be measured by means of DNA band shift assays (also called gel retardation) (Wilson et al. (2001) Journal of Bacteriology 183:2151-2155). The effect of DNA-binding proteins on the expression of other genes can be detected by various well-described methods of reporter gene assay (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

The invention provides replicatable, preferably endogenous nucleotide sequences (DNA) which originate from coryneform bacteria and code for the protein sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid, in particular L-valine, shown in SEQ ID No. 4.

The invention also provides replicatable, preferably endogenous nucleotide sequences (DNA) which originate from coryneform bacteria and code for the protein sigma factor A, the associated base sequence of which contains thymine at position 1241, shown in SEQ ID No. 3.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

The invention also provides vectors (plasmids) which contain the nucleotide sequences mentioned and optionally replicate in coryneform bacteria.

Coryneform bacteria which preferably contain the nucleotide sequence(s) mentioned according to the nucleotide sequences

which code for sigma factor A in an over-expressed form are also claimed.

The invention provides a process for the preparation of L-lysine or feedstuffs additives comprising L-lysine in which in general the following steps are carried out:

a) fermentation of coryneform bacteria which contain endogenous nucleotide sequences which code for sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 is replaced by another proteinogenic amino acid, preferably L-valine,

the alleles of the endogenous sigA gene are over-expressed under conditions suitable for the formation of the sigA gene product sigma factor A,

b) concentration of the L-lysine in the fermentation broth,

c) isolation of the L-lysine or feedstuffs additive comprising L-lysine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass (> 0 to 100%).

Proteinogenic amino acids are to be understood as meaning all amino acids which are constituents of proteins or polypeptides. These are, in particular: L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

The wild-type form of the sigA gene is contained in wild-type strains of coryneform bacteria, in particular of the

genus corynebacterium. It is shown in SEQ ID No. 1. The wild-type protein is shown in SEQ ID No. 2.

Of the genus Corynebacterium, the species Corynebacterium glutamicum known to experts is to be mentioned in particular. Known wild-type strains of the species Corynebacterium glutamicum are, for example

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020.

Strains with the designation "ATCC" can be obtained from the American Type Culture Collection (Manassas, VA, USA). Strains with the designation "FERM" can be obtained from the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba Ibaraki, Japan). The strain of Corynebacterium thermoaminogenes mentioned (FERM BP-1539) and others (FERM BP-1540, FERM BP-1541 and FERM BP-1542) are described in US-A 5,250,434.

To produce the sigA alleles according to the invention which code for variants of sigma factor A characterized by an amino acid exchange at position 414 of SEQ ID No. 2, mutagenesis methods described in the prior art are used.

Conventional in vivo mutagenesis methods using mutagenic substances, such as, for example, N-methyl-N'-nitro-N-nitrosoguanidine, or ultraviolet light can be used for the mutagenesis.

In vitro methods, such as, for example, a treatment with hydroxylamine (Miller, J. H.: A Short Course in Bacterial

Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T. A. Brown: Gentechnologie für Einsteiger [Genetic Engineering for Beginners], Spektrum Akademischer Verlag, Heidelberg, 1993) or the polymerase chain reaction (PCR) such as is described in the handbook by Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994) can furthermore be used for the mutagenesis.

Further instructions on generation of mutations can be found in the prior art and in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

If in vitro methods are used, the sigA gene described in the prior art is amplified starting from isolated complete DNA of a wild-type strain with the aid of the polymerase chain reaction, optionally cloned in suitable plasmid vectors, and the DNA is then subjected to the mutagenesis process. Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994). Suitable sigA alleles are then selected by the processes described above and investigated.

The invention provides a new sigA allele which codes for a variant of sigma factor A and is shown in SEQ ID No. 3.

The sigA alleles according to the invention can be transferred into suitable strains by the method of gene replacement, such as is described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) or Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)). The corresponding sigA allele is cloned here in a vector which is not replicative for C. glutamicum, such as, for example, pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)) or pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) and this is then transferred into the desired host of C. glutamicum by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation is achieved.

In addition, it may be advantageous for the production of L-amino acids at the same time to enhance, in particular over-express one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the use of the sigA allele according to the invention. The use of endogenous genes is in general preferred.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences and alleles thereof present in the population of a species.

The term "enhancement" in this connection describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for

a corresponding enzyme or protein having a high activity, and optionally combining these measures. An increase in the activity of the corresponding enzyme protein can also be effected by a reduced sensitivity to inhibitors.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

Thus, for the preparation of L-lysine, in addition to the use of the variants of the sigA gene, at the same time one or more of the endogenous genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the eno gene which codes for enolase (DE: 19947791.4),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661, EP-A-1108790),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609; EP-A1108790),

- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759; WO 00/63388),
- the lysE gene which codes for the lysine export protein (DE-A-195 48 222),
- the zwa1 gene which codes for the Zwa1 protein (DE: 19959328,0, DSM 13115)
- the gnd gene which codes for 6-phosphogluconate dehydrogenase (WO 01/71012),
- the opcA gene which codes for a sub-unit of glucose 6-phosphate dehydrogenase (sequence no. 79 from WO 01/00844; WO 01/04322),

can be enhanced, in particular over-expressed.

The enhancement of 6-phosphogluconate dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-proline for L-serine, L-leucine, L-isoleucine or L-threonine at position 158 of the enzyme protein and/or by exchange of L-serine for L-phenylalanine or L-tyrosine at position 361 of the enzyme protein.

The enhancement of the glucose 6-phosphate dehydrogenase sub-unit can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-serine by L-phenylalanine or L-tyrosine at position 312 of the enzyme protein.

It may be furthermore advantageous for the production of L-lysine, in addition to the use of the variants of the sigA

gene, at the same time for one or more of the endogenous genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- the zwa2 gene which codes for the Zwa2 protein (DE: 19959327,2, DSM 13113),
- the fda gene which codes for fructose 1,6-bisphosphate aldolase (Accession No. X17313; von der Osten et al., Molecular Microbiology 3 (11), 1625-1637 (1989)),
- the hom gene which codes for homoserine dehydrogenase (EP-A -0131171),
- the leuB gene which codes for isopropyl malate dehydrogenase (Pátek et al., Applied Environmental Microbiology 50:43-47(1989)), Accession No. Y09578),
- the leuC gene which codes for isopropyl malate dehydratase (Accession No. AX121536, sequence no. 1452 from patent EP1108790, Accession No. AX063983, sequence no. 265 from patent WO0100843),
- the thrB gene which codes for homoserine kinase (Peoples, O.W., et al., Molecular Microbiology 2:63-72(1988)) and
- the pfkB gene which codes for phosphofructokinase (SEQ ID No. 57 from WO 01/00844)

to be attenuated, in particular for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme or protein, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

The attenuation of isopropyl malate dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-glycine for L-aspartate, L-asparagine or L-glutamate at position 131 of the enzyme protein.

The attenuation of isopropyl malate dehydratase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-arginine for L-serine at position 451 or L-glycine for L-aspartate at position 456 of the enzyme protein or a combination thereof.

The attenuation of homoserine dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-asparagine for L-threonine or L-serine at position 118 or L-leucine for L-proline at position 160 of the enzyme protein or a combination thereof.

The attenuation of phosphofructokinase can also be achieved, inter alia, by amino acid exchanges, such as, for

example, by exchange of L-leucine for L-alanine, L-glycine or L-proline at position 109 of the enzyme protein.

The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be

used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example

as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for fermentative preparation of L-lysine.

The concentration of L-lysine can optionally be adjusted to the desired value by addition of L-lysine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

Amplification and sequencing of the DNA of the sigA allele of strain DM1547

The Corynebacterium glutamicum strain DM1547 was prepared by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC13032. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and methionine-sensitive.

From the strain DM1547, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA section which carries the sigA gene or allele is amplified. On the basis of the sequence of the sigA gene known for C. glutamicum (sequence no. 2100 and sequence no. 7065 from EP1108970), the following primer oligonucleotides are chosen for the PCR:

sigA-1 (SEQ ID No. 8):
5` tgatcggctgaccaactcta 3`

sigA-2 (SEQ ID No. 9):
5` aaggtctcgaatccgagaac 3`

The primers shown are synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction is carried out by

the standard PCR method of Innis et al. (PCR protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.89 kb in length, which carries the sigA allele.

The amplified DNA fragment of approx. 1.89 kb in length which carries the sigA allele of the strain DM1547 is identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The nucleotide sequence of the amplified DNA fragment or PCR product is determined by sequencing by MWG Biotech (Ebersberg, Germany). The sequence of the PCR product is shown in SEQ ID No. 5. The sequence of the coding region is shown again in SEQ ID No. 3. The amino acid sequences of the associated sigma factor A protein resulting with the aid of the Patentin program are shown in SEQ ID No. 6 and 4.

At position 1241 of the nucleotide sequence of the coding region of the sigA allele of strain DM1547, that is to say at position 1466 of the nucleotide sequence shown in SEQ ID No. 5, is the base thymine. At the corresponding position of the wild-type gene is the base cytosine (SEQ ID No. 1).

At position 414 of the amino acid sequence of sigma factor A of strain DM1547 is the amino acid valine (SEQ ID No. 6 and 4). At the corresponding position of the wild-type protein is the amino acid alanine (SEQ ID No. 2).

The sigA allele, which contains the base thymine at position 1241 of the coding region and accordingly codes for a sigma factor A which contains the amino acid valine at position 414 of the amino acid sequence, is called the sigA_A414V allele in the following. In the designation "sigA_A414V", A represents L-alanine, V represents L-valine

and 414 indicates the position of the amino acid exchange (see SEQ ID No. 2 and 4).

Example 2

Replacement of the sigA wild-type gene of strain DSM5715 by the sigA_A414V allele

2.1. Production of a DNA fragment which carries the region of the sigA_A414V allele on which the mutation A414V is located

From the strain DM1547, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). A DNA section which carries the region of the sigA_A414V allele on which the mutation A414V is located is amplified with the aid of the polymerase chain reaction. On the basis of the sequence of the sigA gene known for C. glutamicum (sequence no. 2100 and sequence no. 7065 from EP-A-1108790), the following primer oligonucleotides are chosen for the PCR such that the mutation A414V is located in the central region of the amplification product:

sigA_XL-A1 (SEQ ID No. 10):
5` ac gaa ttc-cga cgg cga tga ctt cgt ag 3`

sigA_XL-A2 (SEQ ID No. 11):
5` tg gaa ttc-cgt tcc acc tcg ctc cat tc 3`

The primers shown are synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press). The primers allow amplification of a DNA section approx. 1.69 kb in length which carries a region of the sigA_A414V allele (SEQ ID No. 7). The primers moreover contain the sequence for a cleavage site of the restriction

endonuclease EcoRI, which is marked by underlining in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.69 kb in length which carries the sigA allele of the strain DM1547 is cleaved with the restriction endonuclease EcoRI, identified by electrophoresis in a 0.8% agarose gel and then isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

2.2. Construction of the exchange vector pK18mobsacB_sigA_A414V

The approx. 1.68 kb long DNA fragment cleaved with the restriction endonuclease EcoRI, which contains a region of the sigA_A414V allele which carries the mutation A414V, is incorporated by means of replacement mutagenesis with the aid of the sacB system described by Schäfer et al. (Gene, 14, 69-73 (1994)) into the chromosome of the C. glutamicum strain DSM5715. This system enables preparation and selection of allele exchanges which take place by homologous recombination.

The mobilizable cloning vector pK18mobsacB is digested with the restriction enzyme EcoRI and the ends are dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany). The vector prepared in this way is mixed with the sigA_A414V fragment of approx. 1.68 kb and the mixture is treated with T4 DNA ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain S17-1 (Simon et al., Bio/Technologie [Bio/Technology] 1: 784-791, 1993) is then transformed with the ligation batch (Hanahan, In. DNA cloning. A practical approach. Vol.1. ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: a laboratory manual. 2nd Ed.

Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme BamHI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB_sigA_A414V and is shown in figure 1.

2.3 Allele exchange

The vector pK18mobsacB_sigA_A414V mentioned in example 2.2 is transferred by conjugation by a protocol of Schäfer et al. (Journal of Microbiology 172: 1663-1666 (1990)) into the C. glutamicum strain DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it is present integrated in the chromosome as the consequence of a recombination event. Selection of transconjugants, i.e. clones with integrated pK18mobsacB_sigA_A414V, is made by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: a laboratory manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Kanamycin-resistant transconjugants are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 24 hours at 33°C. For selection of mutants in which excision of the plasmid has taken place as a consequence of a second recombination event, the clones are cultured unselectively for 30 hours in LB liquid medium and then plated out on LB agar with 10% sucrose and incubated for 16 hours.

The plasmid pK18mobsacB_sigA_A414V, like the starting plasmid pK18mobsacB, contains, in addition to the kanamycin resistance gene, a copy of the sacB gene which codes for levan sucrase from Bacillus subtilis. The expression which can be induced by sucrose leads to the formation of levan sucrase, which catalyses the synthesis of the product

levan, which is toxic to C. glutamicum. Only those clones in which the integrated pK18mobsacB_sigA_A414V has excised as the consequence of a second recombination event therefore grow on LB agar. Depending on the position of the second recombination event with respect to the mutation site, allele exchange or incorporation of the mutation takes place with the excision, or the original copy remains in the chromosome of the host.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". In 4 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin", a region of the sigA gene spanning the mutation A414V is sequenced, starting from the sequencing primer sA_1 (SEQ ID No. 12), by GATC Biotech AG (Constance, Germany) to demonstrate that the mutation of the sigA_A414V allele is present in the chromosome. The primer sA_1 used is synthesized for this by GATC:

sA_1 (SEQ ID No. 12):
5` aag ttc tcc acc tac gca ac 3`

A clone which contains the base thymine at position 1241 of the sigA gene and thus has the sigA_A414V allele was identified in this manner. This clone was called strain DSM5715sigA_A414V.

Example 3

Preparation of lysine

The C. glutamicum strain DSM5715sigA_A414V obtained in example 2 is cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant is determined.

For this, the strain is first incubated on an agar plate for 24 hours at 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the preculture. The preculture is incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main culture is 0.1. The Medium MM is also used for the main culture.

Medium MM

CSL	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution are

brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, as well as the $CaCO_3$ autoclaved in the dry state, are then added.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in table 1.

Tabelle 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715	8.2	13.57
DSM5715sigA_A414V	8.0	15.21

Brief description of the figure:

Figure 1: Map of the plasmid pK18mobsacB_sigA_A414V.

The abbreviations and designations used have the following meaning. The base pair numbers stated are approximate values obtained in the context of reproducibility of measurements.

Kan:	Kanamycin resistance gene
EcoRI:	Cleavage site of the restriction enzyme EcoRI
BamHI:	Cleavage site of the restriction enzyme BamHI
´sigA:	Cloned DNA fragment containing a 3' terminal region of the sigA allele (= sigA_A414V allele) and the downstream region
sacB:	sacB gene
RP4-mob:	mob region with the replication origin for the transfer (oriT)
oriV:	Replication origin V

WHAT IS CLAIMED IS:

1. Replicatable nucleotide sequences (DNA) which originate from coryneform bacteria and code for the protein sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid.

2. Replicatable nucleotide sequence (DNA) according to claim 1 which originates from coryneform bacteria and codes for the protein sigma factor A, wherein the associated amino acid sequence contains the L-valine at position 414, shown in SEQ ID No. 4.

3. Replicatable nucleotide sequence (DNA) according to claim 1 which originates from coryneform bacteria and codes for the protein sigma factor A, the base sequence of which contains thymine at position 1241, shown in SEQ ID No. 3.

4. Plasmids (vectors) which contain the nucleotide sequences according to claims 1 to 3 and optionally replicate in coryneform bacteria.

5. Coryneform bacteria which contain nucleotide sequences according to claims 1 to 4 and in which the nucleotide sequences which code for sigma factor A are preferably present in over-expressed form.

6. Coryneform bacteria which contain nucleotide sequences which code for sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid.

7. Process for the preparation of L-lysine or feedstuffs additives comprising L-lysine, in which the following steps are carried out (comprising):

a) fermentation of coryneform bacteria in which alleles of the endogenous sigA gene are over-expressed under conditions which are suitable for the formation of the sigA gene product sigma factor A; and

b) isolation of the L-lysine or the feedstuffs additive comprising L-lysine from the fermentation broth, the coryneform bacteria forming the L-lysine.

8. Process according to claim 7, wherein coryneform bacteria which contain an allele of the sigA gene are employed, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid.

9. Process according to claim 7, wherein microorganisms in which further genes of the biosynthesis pathway of L-lysine are additionally over-expressed are employed.

10. Process according to claim 7, wherein microorganisms in which the metabolic pathways which reduce the formation of L-lysine are at least partly eliminated are employed.

11. Process for the preparation of L-lysine or feedstuffs additives comprising L-lysine, in which the following steps are carried out:

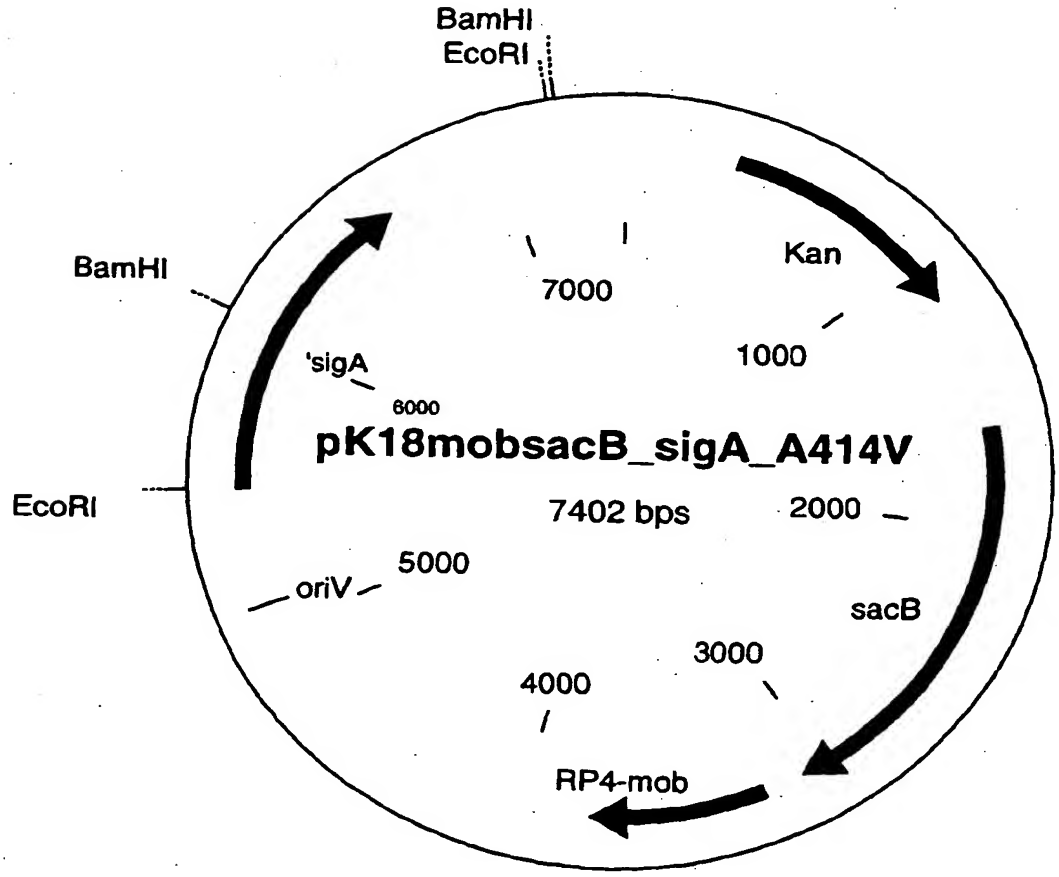
a) fermentation of coryneform bacteria which contain endogenous nucleotide sequences which code for the protein sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 is replaced by another proteinogenic amino acid, preferably L-valine,

b) concentration of the L-lysine in the fermentation broth,

c) isolation of the L-lysine or feedstuffs additive comprising L-lysine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass (> 0 to 100%).

Figure 1: Plasmid pK18mobsacB_sigA_A414V



SEQUENCE LISTING

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Lys Lys Thr Ala Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala
            20                  25                  30

acc cca ttg gga gtc gca tct gag tct ccc att tcg gcc acc cct gcg      144
Thr Pro Leu Gly Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala
        35                  40                  45

cgc agc atc gat gga acc tca acc cct gtt gaa gct gct gac acc ata      192
Arg Ser Ile Asp Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile
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Glu Thr Thr Ala Pro Ala Ala Lys Ala Pro Ala Ala Lys Ala Pro Ala
65                  70                  75                  80

aaa aag gtt gcc aag aag aca gct cgc aag gca cct gcg aaa aag act      288
Lys Lys Val Ala Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr
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gtc gcc aag aaa gcc aca acc gcc aag gct gca cct gca act gcc aag      336
Val Ala Lys Lys Ala Thr Thr Ala Lys Ala Ala Pro Ala Thr Ala Lys
            100                 105                 110

gac gaa aac gca cct gtt gat gac gac gag gag aac ctc gct cag gat      384
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cgt cag gca cgt aaa gat gcc gag ctc acc gct tcc gcc gac tct gtt      576
Arg Gln Ala Arg Lys Asp Ala Glu Leu Thr Ala Ser Ala Asp Ser Val
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cgc gct tac ctg aag caa atc ggt aaa gtt gcc ctg ctg aac gct gaa      624
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Lys Lys Thr Ala Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala
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Thr Pro Leu Gly Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala
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Arg Ser Ile Asp Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile
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Lys Lys Val Ala Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr
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Val Ala Lys Lys Ala Thr Thr Ala Lys Ala Ala Pro Ala Thr Ala Lys
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gaa gag gac ggc tca tcc gtt tgg gat gaa gac gaa tcc gca acc ctg      528
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cgt cag gca cgt aaa gat gcc gag ctc acc gct tcc gcc gac tct gtt      576
Arg Gln Ala Arg Lys Asp Ala Glu Leu Thr Ala Ser Ala Asp Ser Val
            180                 185                 190

cgc gct tac ctg aag caa atc ggt aaa gtt gcc ctg ctg aac gct gaa      624
Arg Ala Tyr Leu Lys Gln Ile Gly Lys Val Ala Leu Leu Asn Ala Glu
        195                 200                 205

cag gaa gtc tcc ctg gca aag cgc atc gaa gca ggc ctt tac gcc acc      672
Gln Glu Val Ser Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr Ala Thr
    210                 215                 220

cac cgc atg gag gaa atg gaa gaa gct ttc gca gcc ggt gac aag gac      720
His Arg Met Glu Glu Met Glu Glu Ala Phe Ala Ala Gly Asp Lys Asp
225                 230                 235                 240

gcg aaa ctc acc cca gcc gtc aag cgt gac ctc cgc gcc atc gct cgt      768
Ala Lys Leu Thr Pro Ala Val Lys Arg Asp Leu Arg Ala Ile Ala Arg
                245                 250                 255

gac ggc cgc aag gcg aaa aac cac ctc ctg gaa gcc aac ctt cgt ctg      816
Asp Gly Arg Lys Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg Leu
            260                 265                 270

gtt gtc tcc ctg gca aag cgc tac acc ggc cgt ggc atg gca ttc ctg      864
Val Val Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe Leu
        275                 280                 285

gac ctc atc cag gaa ggc aac ctc ggt ctg att cgt gcc gta gag aag      912
Asp Leu Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu Lys
    290                 295                 300

ttc gac tac tcc aag ggc tac aag ttc tcc acc tac gca acc tgg tgg      960
Phe Asp Tyr Ser Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp Trp
305                 310                 315                 320

atc cgt cag gca atc acc cgc gcc atg gcc gac caa gca cga acc atc     1008
Ile Arg Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr Ile
                325                 330                 335

cgt atc cca gtc cac atg gtt gaa gtg atc aac aaa ctt ggt cgc atc     1056
Arg Ile Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg Ile
            340                 345                 350

caa cgt gaa ctc ctt cag gaa ctc ggc cgc gaa cca acc cca cag gaa     1104
Gln Arg Glu Leu Leu Gln Glu Leu Gly Arg Glu Pro Thr Pro Gln Glu
        355                 360                 365

ctg tcc aaa gaa atg gac atc tcc gag gaa aag gta ctg gaa atc cag     1152
Leu Ser Lys Glu Met Asp Ile Ser Glu Glu Lys Val Leu Glu Ile Gln
    370                 375                 380
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cag tac gcc cgc gaa cca atc tcc ctg gac caa acc atc ggc gac gaa      1200
Gln Tyr Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu
385                 390                 395                 400

ggc gac agc cag ctc ggc gac ttc atc gaa gac tcc gaa gtc gtc gtc      1248
Gly Asp Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Val Val Val
                405                 410                 415

gca gtc gac gcc gtc tca ttc acc ctg ctg caa gac cag cta cag gac      1296
Ala Val Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Asp
            420                 425                 430

gtc cta gag acc ctc tcc gaa cgt gaa gcc ggc gtg gtt aaa ctc cgc      1344
Val Leu Glu Thr Leu Ser Glu Arg Glu Ala Gly Val Val Lys Leu Arg
        435                 440                 445

ttc gga ctc acc gac gga atg cca cgc act tta gac gaa atc ggc caa      1392
Phe Gly Leu Thr Asp Gly Met Pro Arg Thr Leu Asp Glu Ile Gly Gln
    450                 455                 460

gtt tac ggt gtc acc cgt gag cgc atc cgc cag att gag tcc aag acc      1440
Val Tyr Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys Thr
465                 470                 475                 480

atg tct aag ctg cgc cac cca tca cgc tcc cag gtc ctt cgc gac tac      1488
Met Ser Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp Tyr
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ctg gac taa                                                         1497
Leu Asp
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<210> 4
<211> 498
<212> PRT
<213> Corynebacterium glutamicum

<400> 4

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Lys Lys Thr Ala Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala
            20                  25                  30

Thr Pro Leu Gly Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala
        35                  40                  45

Arg Ser Ile Asp Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile
    50                  55                  60

Glu Thr Thr Ala Pro Ala Ala Lys Ala Pro Ala Ala Lys Ala Pro Ala
65                  70                  75                  80

Lys Lys Val Ala Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr
                85                  90                  95

Val Ala Lys Lys Ala Thr Thr Ala Lys Ala Ala Pro Ala Thr Ala Lys
            100                 105                 110

Asp Glu Asn Ala Pro Val Asp Asp Asp Glu Glu Asn Leu Ala Gln Asp
        115                 120                 125
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130 135 140

Glu Asp Glu Asp Gly Val Glu Ala Leu Gly Glu Glu Ser Glu Asp Asp
145 150 155 160

Glu Glu Asp Gly Ser Ser Val Trp Asp Glu Asp Glu Ser Ala Thr Leu
165 170 175

Arg Gln Ala Arg Lys Asp Ala Glu Leu Thr Ala Ser Ala Asp Ser Val
180 185 190

Arg Ala Tyr Leu Lys Gln Ile Gly Lys Val Ala Leu Leu Asn Ala Glu
195 200 205

Gln Glu Val Ser Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr Ala Thr
210 215 220

His Arg Met Glu Glu Met Glu Glu Ala Phe Ala Ala Gly Asp Lys Asp
225 230 235 240

Ala Lys Leu Thr Pro Ala Val Lys Arg Asp Leu Arg Ala Ile Ala Arg
245 250 255

Asp Gly Arg Lys Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg Leu
260 265 270

Val Val Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe Leu
275 280 285

Asp Leu Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu Lys
290 295 300

Phe Asp Tyr Ser Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp Trp
305 310 315 320

Ile Arg Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr Ile
325 330 335

Arg Ile Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg Ile
340 345 350

Gln Arg Glu Leu Leu Gln Glu Leu Gly Arg Glu Pro Thr Pro Gln Glu
355 360 365

Leu Ser Lys Glu Met Asp Ile Ser Glu Glu Lys Val Leu Glu Ile Gln
370 375 380

Gln Tyr Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu
385 390 395 400

Gly Asp Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Val Val Val
405 410 415

Ala Val Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Asp
420 425 430

Val Leu Glu Thr Leu Ser Glu Arg Glu Ala Gly Val Val Lys Leu Arg
435 440 445

Phe Gly Leu Thr Asp Gly Met Pro Arg Thr Leu Asp Glu Ile Gly Gln
450 455 460

```
Val Tyr Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys Thr
465                 470                 475                 480

Met Ser Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp Tyr
                485                 490                 495

Leu Asp
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<210> 5
<211> 1892
<212> DNA
<213> Corynebacterium glutamicum

<220>
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<223> sigA allele

<220>
<221> mutation
<222> (1466)..(1466)
<223> Exchange of cytosine for thymine

<400> 5

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ttctggggaa caaatacgtt tccctattgt tgtatatagg tattcgcact taagaaacat    120

ctctcatgga aagaagctag gcggaaaggg cgttaagtac ttgccattta atcctcagca    180

tcactcggat cagtcggaga tgtcgatgaa aatgcaccag gagcc gtg gag agc agc    237
                                                 Val Glu Ser Ser
                                                 1

atg gta gaa aac aac gta gca aaa aag acg gtc gct aaa aag acc gca      285
Met Val Glu Asn Asn Val Ala Lys Lys Thr Val Ala Lys Lys Thr Ala
5                   10                  15                  20

cgc aag acc gca cgc aaa gca gcc ccg cgc gtg gca acc cca ttg gga      333
Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala Thr Pro Leu Gly
                25                  30                  35

gtc gca tct gag tct ccc att tcg gcc acc cct gcg cgc agc atc gat      381
Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala Arg Ser Ile Asp
            40                  45                  50

gga acc tca acc cct gtt gaa gct gct gac acc ata gag acc acc gcc      429
Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile Glu Thr Thr Ala
        55                  60                  65

cct gca gcg aag gct cct gcg gcc aag gct ccc gct aaa aag gtt gcc      477
Pro Ala Ala Lys Ala Pro Ala Ala Lys Ala Pro Ala Lys Lys Val Ala
    70                  75                  80

aag aag aca gct cgc aag gca cct gcg aaa aag act gtc gcc aag aaa      525
Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr Val Ala Lys Lys
85                  90                  95                  100
```

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gcc aca acc gcc aag gct gca cct gca act gcc aag gac gaa aac gca      573
Ala Thr Thr Ala Lys Ala Ala Pro Ala Thr Ala Lys Asp Glu Asn Ala
                105                 110                 115

cct gtt gat gac gac gag gag aac ctc gct cag gat gaa cag gac ttc      621
Pro Val Asp Asp Asp Glu Glu Asn Leu Ala Gln Asp Glu Gln Asp Phe
            120                 125                 130

gac ggc gat gac ttc gta gac ggc atc gaa gac gaa gaa gat gaa gac      669
Asp Gly Asp Asp Phe Val Asp Gly Ile Glu Asp Glu Glu Asp Glu Asp
        135                 140                 145

ggc gtc gaa gcc ctc ggt gaa gaa agc gaa gac gac gaa gag gac ggc      717
Gly Val Glu Ala Leu Gly Glu Glu Ser Glu Asp Asp Glu Glu Asp Gly
    150                 155                 160

tca tcc gtt tgg gat gaa gac gaa tcc gca acc ctg cgt cag gca cgt      765
Ser Ser Val Trp Asp Glu Asp Glu Ser Ala Thr Leu Arg Gln Ala Arg
165                 170                 175                 180

aaa gat gcc gag ctc acc gct tcc gcc gac tct gtt cgc gct tac ctg      813
Lys Asp Ala Glu Leu Thr Ala Ser Ala Asp Ser Val Arg Ala Tyr Leu
                185                 190                 195

aag caa atc ggt aaa gtt gcc ctg ctg aac gct gaa cag gaa gtc tcc      861
Lys Gln Ile Gly Lys Val Ala Leu Leu Asn Ala Glu Gln Glu Val Ser
            200                 205                 210

ctg gca aag cgc atc gaa gca ggc ctt tac gcc acc cac cgc atg gag      909
Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr Ala Thr His Arg Met Glu
        215                 220                 225

gaa atg gaa gaa gct ttc gca gcc ggt gac aag gac gcg aaa ctc acc      957
Glu Met Glu Glu Ala Phe Ala Ala Gly Asp Lys Asp Ala Lys Leu Thr
    230                 235                 240

cca gcc gtc aag cgt gac ctc cgc gcc atc gct cgt gac ggc cgc aag     1005
Pro Ala Val Lys Arg Asp Leu Arg Ala Ile Ala Arg Asp Gly Arg Lys
245                 250                 255                 260

gcg aaa aac cac ctc ctg gaa gcc aac ctt cgt ctg gtt gtc tcc ctg     1053
Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg Leu Val Val Ser Leu
                265                 270                 275

gca aag cgc tac acc ggc cgt ggc atg gca ttc ctg gac ctc atc cag     1101
Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe Leu Asp Leu Ile Gln
            280                 285                 290

gaa ggc aac ctc ggt ctg att cgt gcc gta gag aag ttc gac tac tcc     1149
Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu Lys Phe Asp Tyr Ser
        295                 300                 305

aag ggc tac aag ttc tcc acc tac gca acc tgg tgg atc cgt cag gca     1197
Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp Trp Ile Arg Gln Ala
    310                 315                 320

atc acc cgc gcc atg gcc gac caa gca cga acc atc cgt atc cca gtc     1245
Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr Ile Arg Ile Pro Val
325                 330                 335                 340
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cac atg gtt gaa gtg atc aac aaa ctt ggt cgc atc caa cgt gaa ctc      1293
His Met Val Glu Val Ile Asn Lys Leu Gly Arg Ile Gln Arg Glu Leu
                345                 350                 355

ctt cag gaa ctc ggc cgc gaa cca acc cca cag gaa ctg tcc aaa gaa      1341
Leu Gln Glu Leu Gly Arg Glu Pro Thr Pro Gln Glu Leu Ser Lys Glu
            360                 365                 370

atg gac atc tcc gag gaa aag gta ctg gaa atc cag cag tac gcc cgc      1389
Met Asp Ile Ser Glu Glu Lys Val Leu Glu Ile Gln Gln Tyr Ala Arg
        375                 380                 385

gaa cca atc tcc ctg gac caa acc atc ggc gac gaa ggc gac agc cag      1437
Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu Gly Asp Ser Gln
    390                 395                 400

ctc ggc gac ttc atc gaa gac tcc gaa gtc gtc gtc gca gtc gac gcc      1485
Leu Gly Asp Phe Ile Glu Asp Ser Glu Val Val Val Ala Val Asp Ala
405                 410                 415                 420

gtc tca ttc acc ctg ctg caa gac cag cta cag gac gtc cta gag acc      1533
Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Asp Val Leu Glu Thr
                425                 430                 435

ctc tcc gaa cgt gaa gcc ggc gtg gtt aaa ctc cgc ttc gga ctc acc      1581
Leu Ser Glu Arg Glu Ala Gly Val Val Lys Leu Arg Phe Gly Leu Thr
            440                 445                 450

gac gga atg cca cgc act tta gac gaa atc ggc caa gtt tac ggt gtc      1629
Asp Gly Met Pro Arg Thr Leu Asp Glu Ile Gly Gln Val Tyr Gly Val
        455                 460                 465

acc cgt gag cgc atc cgc cag att gag tcc aag acc atg tct aag ctg      1677
Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys Thr Met Ser Lys Leu
    470                 475                 480

cgc cac cca tca cgc tcc cag gtc ctt cgc gac tac ctg gac              1719
Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp Tyr Leu Asp
485                 490                 495

taaaacccca gtcgggctca agaccgggcc gccactgttt tcctctgcgg ggaacggtgg   1779

tggcccggtt tttctgttgc tttggttcgg ctggtcacag ttcggctggg gtgttttaag   1839

tttgatttca cattgccgat ttctaaacgc cgagttctcg gattcgagac ctt          1892
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<210> 6
<211> 498
<212> PRT
<213> Corynebacterium glutamicum

<400> 6

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1               5                   10                  15

Lys Lys Thr Ala Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala
            20                  25                  30

Thr Pro Leu Gly Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala
        35                  40                  45
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Arg Ser Ile Asp Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile
    50                  55                  60

Glu Thr Thr Ala Pro Ala Ala Lys Ala Pro Ala Ala Lys Ala Pro Ala
65                  70                  75                  80

Lys Lys Val Ala Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr
                85                  90                  95

Val Ala Lys Lys Ala Thr Thr Ala Lys Ala Ala Pro Ala Thr Ala Lys
            100                 105                 110

Asp Glu Asn Ala Pro Val Asp Asp Asp Glu Glu Asn Leu Ala Gln Asp
        115                 120                 125

Glu Gln Asp Phe Asp Gly Asp Asp Phe Val Asp Gly Ile Glu Asp Glu
    130                 135                 140

Glu Asp Glu Asp Gly Val Glu Ala Leu Gly Glu Glu Ser Glu Asp Asp
145                 150                 155                 160

Glu Glu Asp Gly Ser Ser Val Trp Asp Glu Asp Glu Ser Ala Thr Leu
                165                 170                 175

Arg Gln Ala Arg Lys Asp Ala Glu Leu Thr Ala Ser Ala Asp Ser Val
            180                 185                 190

Arg Ala Tyr Leu Lys Gln Ile Gly Lys Val Ala Leu Leu Asn Ala Glu
        195                 200                 205

Gln Glu Val Ser Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr Ala Thr
    210                 215                 220

His Arg Met Glu Glu Met Glu Glu Ala Phe Ala Ala Gly Asp Lys Asp
225                 230                 235                 240

Ala Lys Leu Thr Pro Ala Val Lys Arg Asp Leu Arg Ala Ile Ala Arg
                245                 250                 255

Asp Gly Arg Lys Ala Lys Asn His Leu Leu Glu Ala Asn Leu Arg Leu
            260                 265                 270

Val Val Ser Leu Ala Lys Arg Tyr Thr Gly Arg Gly Met Ala Phe Leu
        275                 280                 285

Asp Leu Ile Gln Glu Gly Asn Leu Gly Leu Ile Arg Ala Val Glu Lys
    290                 295                 300

Phe Asp Tyr Ser Lys Gly Tyr Lys Phe Ser Thr Tyr Ala Thr Trp Trp
305                 310                 315                 320

Ile Arg Gln Ala Ile Thr Arg Ala Met Ala Asp Gln Ala Arg Thr Ile
                325                 330                 335

Arg Ile Pro Val His Met Val Glu Val Ile Asn Lys Leu Gly Arg Ile
            340                 345                 350

Gln Arg Glu Leu Leu Gln Glu Leu Gly Arg Glu Pro Thr Pro Gln Glu
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Leu Ser Lys Glu Met Asp Ile Ser Glu Glu Lys Val Leu Glu Ile Gln
    370                 375                 380
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Gln Tyr Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu
385 390 395 400

Gly Asp Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Val Val Val
405 410 415

Ala Val Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Asp
420 425 430

Val Leu Glu Thr Leu Ser Glu Arg Glu Ala Gly Val Val Lys Leu Arg
435 440 445

Phe Gly Leu Thr Asp Gly Met Pro Arg Thr Leu Asp Glu Ile Gly Gln
450 455 460

Val Tyr Gly Val Thr Arg Glu Arg Ile Arg Gln Ile Glu Ser Lys Thr
465 470 475 480

Met Ser Lys Leu Arg His Pro Ser Arg Ser Gln Val Leu Arg Asp Tyr
485 490 495

Leu Asp

<210> 7
<211> 1689
<212> DNA
<213> Artificial sequence

<220>
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<222> (1)..(1689)
<223> Description of the artificial sequence: PCR product containing an n 3'-terminal region of the sigA allele (= sigA_A414V allele) and the downstream region

<220>
<221> mutation
<222> (854)..(854)
<223> Exchange of cytosine for thymine

<400> 7
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gaagacgaat ccgcaaccct gcgtcaggca cgtaaagatg ccgagctcac cgcttccgcc 180

gactctgttc gcgcttacct gaagcaaatc ggtaaagttg ccctgctgaa cgctgaacag 240

gaagtctccc tggcaaagcg catcgaagca ggcctttacg ccacccaccg catggaggaa 300

atggaagaag ctttcgcagc cggtgacaag gacgcgaaac tcaccccagc cgtcaagcgt 360

gacctccgcg ccatcgctcg tgacggccgc aaggcgaaaa accacctcct ggaagccaac 420

cttcgtctgg ttgtctccct ggcaaagcgc tacaccggcc gtggcatggc attcctggac 480

ctcatccagg aaggcaacct cggtctgatt cgtgccgtag agaagttcga ctactccaag 540

ggctacaagt tctccaccta cgcaacctgg tggatccgtc aggcaatcac ccgcgccatg 600

gccgaccaag cacgaaccat ccgtatccca gtccacatgg ttgaagtgat caacaaactt 660

ggtcgcatcc aacgtgaact ccttcaggaa ctcggccgcg aaccaacccc acaggaactg 720

tccaaagaaa tggacatctc cgaggaaaag gtactggaaa tccagcagta cgcccgcgaa 780

ccaatctccc tggaccaaac catcggcgac gaaggcgaca gccagctcgg cgacttcatc 840

gaagactccg aagtcgtcgt cgcagtcgac gccgtctcat tcaccctgct gcaagaccag 900

ctacaggacg tcctagagac cctctccgaa cgtgaagccg gcgtggttaa actccgcttc 960

ggactcaccg acggaatgcc acgcacttta gacgaaatcg gccaagttta cggtgtcacc 1020

cgtgagcgca tccgccagat tgagtccaag accatgtcta agctgcgcca cccatcacgc 1080

tcccaggtcc ttcgcgacta cctggactaa aaccccagtc gggctcaaga ccgggccgcc 1140

actgttttcc tctgcgggga acggtggtgg cccggttttt ctgttgcttt ggttcggctg 1200

gtcacagttc ggctggggtg ttttaagttt gatttcacat tgccgatttc taaacgccga 1260

gttctcggat tcgagacctt acctgcaatt tcacggttac aatttctcgt tagcactttc 1320

gcgtactcaa tttcttatgt tcaatttcgg tcggaaaagt gccattttcc gacatcgcct 1380

tgagaaattg aatacaagaa attgagcgca aggtatcgaa cttgagaaat tgagctttag 1440

cactcactcc cctgtttgat gaagtcagca aagccaaaag acgacttcac atctccgact 1500

tcttaacagc gacttctcgt ggctgacttc gctacctaaa cctgagtttc cttagcgaag 1560

tcgtgtgatg agaagtcgtg tgatgagaag ttgggtatga gaagtcagca cacgtgaagt 1620

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ggaattcca 1689

<210> 8
<211> 20
<212> DNA
<213> Artificial sequence

<220>
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<222> (1)..(20)
<223> Description of the artificial sequence: primer sigA-1

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<210> 9
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<221> misc_feature
<222> (1)..(20)
<223> Description of the artificial sequence: primer sigA-2

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aaggtctcga atccgagaac 20

<210> 10
<211> 28
<212> DNA
<213> Artificial sequence

<220>
<221> misc_feature
<222> (1)..(28)
<223> Description of the artificial sequence: primer sigA_XL-A1

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<210> 11
<211> 28
<212> DNA
<213> Artificial sequence

<220>
<221> misc_feature
<222> (1)..(28)
<223> Description of the artificial sequence: primer sigA_XL-A2

<400> 11
tggaattccg ttccacctcg ctccattc 28

<210> 12
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<221> misc_feature
<222> (1)..(20)
<223> Description of the artificial sequence: primer sA_1

<400> 12
aagttctcca cctacgcaac
20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/11561

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/12 C12N15/31 C12N15/52 C07K14/34 C07K14/345
C12N15/77 C12N1/21 C12P13/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 108 790 A (KYOWA HAKKO KOGYO KK) 20 June 2001 (2001-06-20) ---	
A	WO 01 66573 A (BASF AG) 13 September 2001 (2001-09-13) ---	
A	HALGASOVA NORA ET AL: "Cloning and transcriptional characterization of two sigma factor genes, sigA and sigB, from Brevibacterium flavum." CURRENT MICROBIOLOGY, vol. 43, no. 4, October 2001 (2001-10), pages 249-254, XP002227863 ISSN: 0343-8651 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 2003	05/02/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Marinoni, J-C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/11561

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GOMEZ MANUEL ET AL: "sigA Is an essential gene in Mycobacterium smegmatis." MOLECULAR MICROBIOLOGY, vol. 29, no. 2, July 1998 (1998-07), pages 617-628, XP002227864 ISSN: 0950-382X	
A	OGUIZA J A ET AL: "Multiple sigma factor genes in brevibacterium lactofermentum: characterization of sigA and sigB" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 178, no. 2, January 1996 (1996-01), pages 550-553, XP002183859 ISSN: 0021-9193	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/11561

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1108790 A	20-06-2001	EP 1108790 A2	20-06-2001
		JP 2002191370 A	09-07-2002
		US 2002197605 A1	26-12-2002
WO 0166573 A	13-09-2001	AU 2390301 A	17-09-2001
		EP 1257649 A2	20-11-2002
		EP 1261718 A2	04-12-2002
		WO 0100843 A2	04-01-2001
		WO 0166573 A2	13-09-2001